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TiO_2-Soybean Peroxidase composite materials as a new photocatalytic system

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Abstract

In the present work, we prepared a new photoactive system, able to remove water pollutants by dispersion of TiO_2 and the enzyme soybean peroxidase (SBP) within a polymer matrix. UV-cured acrylic polymeric films were prepared, in the presence of TiO_2 and SBP. The choice of SBP was based on its ability to catalyze the oxidation of several substrates, coupled with a strong stability and thermal resistance. Since SBP requires H_2O_2 as cosubstrate, we verified the TiO_2 ability to produce, under irradiation, enough H_2O_2 to activate SBP, both in aqueous suspension and when dispersed in the polymeric film. The experimental data support the possibility of using only the H_2O_2 produced by TiO_2, thus allowing to devise a system able to be activated only by irradiation and self-renew, without the addition of reagents from the outside.

The efficiency of the TiO_2/SBP system, both dispersed in film and in aqueous suspension, were tested on 2,4,6-trichlorophenol. It was observed that TiO_2 and SBP act synergistically, leading to an increase in the rate of removal of the model molecule. In addition, the system exhibits ~~a~~ certain selectivity on the intermediates products, above all hindering the formation of chloroderivatives, which is particularly interesting for future application in the field of environmental purification.

Keywords

Titanium dioxide, soybean peroxidase, UV-curing, trichlorophenol.

1. Introduction

Heterogeneous photocatalysis is an advanced oxidation process that exploits the characteristics of some solids semiconductors, which by means of the absorption of photons assume the function of photocatalysts [1-3] that lead to the decomposition of organic substances and inorganic pollutants [4-10]. This technique is widely employed in the degradation of water pollutants and allows to obtain in many cases the complete mineralization to CO_2, H_2O and inorganic salts [11-13]. In the present work, we have investigated the possibility to obtain a new photoactive system, able to remove pollutants present in water, using a photoactive semiconductor oxide (titanium dioxide) and the enzyme soybean peroxidase (SBP).

As semiconductor, TiO_2 is widely used since it is not toxic, very resistant to photocorrosion and inexpensive; moreover, it can be used at room temperature and ensures high efficiency of degradation. It is also known that titanium dioxide, under irradiation in aqueous solution, is capable of producing hydrogen peroxide at concentration level in the micromolar range [14-16]. In the presence of oxygen, hydrogen peroxide could be produced through the reactions 1-3:

$$e^-_{CB} + O_2 \rightarrow O_2^{\cdot -} \quad (1)$$

$$O_2^{\cdot -} + H^+ \rightarrow HO_2^{\cdot} \quad (2)$$

$$HO_2^{\cdot} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad (3)$$

Hydrogen peroxide action can be increased by enzymatic activation in the presence of a peroxidase, such as SBP. Soybean peroxidase is a plant glycoprotein containing a Fe(III)-heme protoporphyrin IX prosthetic group as active site. Plant peroxidases are implicated *in vivo* in the lignification cycle; their catalytic cycle involves the two-electron reduction of

H_2O_2, followed by the one-electron oxidation of two substrate molecules (SH) *via* two intermediates (Compound I and Compound II), as summarized in the reactions 4-6:

$$SBP + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (4)$$

$$\text{Compound I} + SH \rightarrow \text{Compound II} + S^{\bullet} \quad (5)$$

$$\text{Compound II} + SH \rightarrow SBP + S^{\bullet} + H_2O \quad (6)$$

Such mechanism allows the oxidation of inorganic and organic substrates, including important environmental pollutants such as dyes or polychlorophenols [17-19]. In addition, SBP shows a remarkable thermal stability, works well in a wide pH range and has a low sensitivity to various organic solvents [20-21]. These features ensure the possibility of creating a very stable system that could be self-maintained by the TiO_2 action.

From a practical point of view, the possibility to have the photocatalyst in film form rather than a powder dispersion into aqueous solution, will make it more viable due to its easy separation recovery at the end of the process. For this reason, in our previous investigations we have dispersed photocatalysts, i.e. TiO_2 [22], SWCNTs [23] and GOx [24], into UV-cured epoxy films and we have proved their efficiency in the photodegradation of target molecules.

In this work we have evaluated the possibility of using the TiO_2/SBP system dispersed on a polymer film, which would reduce the costs of disposal and recovery of the catalytic material. Herein, we report the preparation of UV-cured films containing TiO_2 and SBP and their performance in photocatalytic applications. The use of the UV-curing method to prepare the filled polymeric material is particularly attractive since it is an environmental friendly technique, in which the cured film is prepared starting from acrylic monomer in the presence of a suitable radical photoinitiator. The curing process is fast, occurs at room temperature and in the absence of any solvents; by UV irradiation it is possible to induce

the polymer formation with a fast transformation of the liquid monomer into a solid film with tailored physical-chemical and mechanical properties [25].

Firstly, we have tested the ability of TiO_2, both in aqueous suspension and dispersed in the polymeric film, to produce enough H_2O_2 to activate SBP and assessed if the enzyme does not lose its activity when dispersed in the polymer film or subjected to irradiation. Then, the ability of the TiO_2/SBP system to abate pollutants was evaluated using 2,4,6-trichlorophenol as target molecule, and, finally, the main intermediate reaction products obtained both with the suspension and the film were also identified.

2 Materials and methods

2.1 Materials

Phenol, 4-aminoantipyrine, catechol, 1,4-benzoquinone, 2,4,6-trichlorophenol (2,4,6-TCP), 4-chlorophenol, 2,6-dichlorophenol, 3-methyl-2-benzothiazolinone (MBTH), 3-(dimethylamino)benzoic acid (DMAB), sodium dihydrogenphosphate and hydrogen peroxide were all purchased from Aldrich. Acetonitrile (AC0331 Supergradient HPLC grade eluent) was purchased from Scharlau.

Soybean peroxidase (SBP) was furnished by Bio-Research Products Inc., Iowa-USA.

Titanium dioxide was supplied by Degussa (TiO_2, P25, Degussa, Germany, average particles size of 21 nm, specific surface area of 50 m^2/g). The 2-hydroxy-2-methyl-1-phenylpropan-1-one (DAROCUR 1173, BASF) was used as radical photoinitiator.

All aqueous solutions were prepared with ultrapure water Millipore Milli-Q™.

2.2 Preparation of polymeric film

For photocatalytic study on UV cured film, the TiO_2 ceramic filler was added to the polyethyleneglicoldiacrylate (PEGDA) at a content of 3 wt% either with or without SBP (at

a content in the range from 0.2 to 2 wt%). The mixtures were stirred with Ultraturrax until a uniform dispersion was achieved. The photoinitiatior DAROCUR 1173 was added at 2 wt% with respect to the acrylic monomer. The formulations were coated on glass substrate with a wirewound applicator and UV irradiated. Cured films of 100 μm thickness were achieved.

2.3 Polymeric films characterizations

The photopolymerization kinetics were determined by Real-Time FT-IR spectroscopy, employing a Thermo-Nicolet 5700 instrument. The formulations were coated onto a silicon wafer. By real time FT-IR it is possible to follow the photopolymerization process during irradiation. The sample was simultaneously UV-irradiated and analyzed *in-situ* by FT-IR. A mercury lamp medium pressure (Helios italquartz UV-prayer) was used to induce the photopolymerization (light intensity on the surface of the sample was about 30 mW/cm^2). The gel content was determined on the cured films by measuring the weight loss after 24 hours extraction with chloroform at room temperature, according to the standard test method ASTM D2765-84.

The morphology of the obtained materials was investigated by means of a Field Emission Scanning Electron Microscopy (FESEM, Supra 40 Zeiss) performed on the surface fracture of the cured coatings.

2.4. Irradiation procedures

2,4,6-Trichlorophenol was used as target molecule to investigate the photocatalytic effect of the UV-cured material. The irradiation was performed by using a TL K05 UV/A lamp 25 mW/m^2 centered at 365 nm and the experiments were carried out in stirred Pyrex glass cells filled with 5 mL of sample for experiments in the presence of TiO_2 suspension, or 30 mL when using polymeric films.

2,4,6-TCP (8×10^{-5} M) was irradiated in the presence of: (1) SBP (1×10^{-8} M) and H_2O_2 (1×10^{-4} M); (2) dispersed TiO_2 (1 g L^{-1}); (3) dispersed TiO_2 (1 g L^{-1}) and SBP (1×10^{-8} M). For the irradiation experiments carried out in cells containing polymeric films, the cured films containing 3 wt% of the semiconductor and SBP were dipped into the solution. The different systems studied are: (1) TiO_2 (3 wt%); (2) SBP (1 wt%) and H_2O_2 (1×10^{-4} M); (3) TiO_2 (3 wt%) and SBP (concentrations ranging from 0.2 to 1 wt%).

In all cases, experiments were run without modifying the natural pH of the solution. All experiments were performed in triplicate and the average results are plotted in the graphs.

2.5. Samples analysis

Hydrogen peroxide was quantified by a spectrophotometric assay based on a magenta dye formation in the presence of 4-aminoantipyrine and phenol [26], and the absorbance was measured through a UV-Vis Varian Cary spectrophotometer.

Enzyme activity was measured at different H_2O_2 concentrations through a spectrophotometric method which takes advantage of the reaction between DMAB (5.0×10^{-4} M) and MBTH (2.1×10^{-5} M) with production of a blue indamine dye, which has an absorbance maximum at 590 nm (ε_{590} = 47600 M^{-1} cm^{-1}) [27].

The disappearance of 2,4,6-TCP as a function of the irradiation time and the evaluation of intermediates were followed using an HPLC system (Merck-Hitachi L-6200 pumps), equipped with a Rheodyne injector, a RP C18 column (Lichrochart, Merck, 12.5 cm × 0.4 cm, 5 μm packing) and a UV-Vis detector (Merck Hitachi L-4200). Elution was carried out with acetonitrile and phosphate buffer (1×10^{-2} M) at pH 2.8 (40:60 % v/v) at a flow rate of 1 mL/min. The analytical detector wavelength was 220 nm.

The intermediate products were determined by comparison with standard solutions. In the specific, the identification was carried out on the basis of the retention times, and the quantification was obtained by external calibration curves performed for each standard.

3. Results and discussion

3.1. Hybrid films preparation and characterization

UV-cured PEGDA films were prepared starting from the acrylic monomer in the presence of TiO_2, SBP and the suitable photoinitiator. The main concern on the use of the UV curing technique for the preparation of this new material was the well-known UV absorption property of anatase which could hinder the photoinitiator decomposition, so affecting the efficiency of the radical chain grown polymerization. For this reason, the curing process was investigated by means of RT-FTIR analyses, following the decrease of the acrylic double bond band at around 1640 cm^{-1} both for the pristine acrylic resin and for the resin containing the ceramic filler.

The conversion curves (not reported) show that, in the range of the investigated formulations, the increasing content of TiO_2 does not significantly affect the UV-curing process. When TiO_2 is added to the formulation, the rate of acrylic group conversion was only slightly decreased. The slight lowered rate of polymerization and acrylic group conversion could be due to the UV light shielding effect of TiO_2 nanoparticles. This competitive effect of UV absorption with the photoinitiator will generate a lower amount of reactive species with a decrease of the acrylic group conversion. In any case, the shielding effect is really very limited because of the very low TiO_2 content in the photocurable formulation. The crosslinked films showed always very high gel content values, indicating the absence of any extractable monomer or oligomers.

Morphological analysis of the achieved UV cured coatings containing titanium dioxide was performed by FESEM on the criofracture surface of the cured films. The FESEM image is reported in Figure 1. It is possible to observe that the TiO_2 particles are homogeneously distributed within the matrix. The particles dimension ranged from 30-40 nm up to aggregation in the order of 100-200 nm.

Figure 1

3.2. Evaluation of enzymatic activity as a function of H_2O_2 produced by TiO_2 irradiation

The first phase of the study has been directed to evaluate the amount of H_2O_2 produced by TiO_2 under irradiation, and whether it was adequate to activate SBP.

Formation of hydrogen peroxide during the irradiation was evaluated both for films immersed in water and for powder suspensions containing increasing amount of TiO_2. The relative hydrogen peroxide concentrations are plotted in Figure 2 as a function of the irradiation time. The production of H_2O_2 increases when enhancing concentration of titanium dioxide and the irradiation time. Considering the TiO_2 suspensions, about 1 μM H_2O_2 is produced within 60 minutes of irradiation from 1 g L^{-1} TiO_2; higher concentrations of TiO_2 were not considered since the photodegradation efficiency levels off [28] at high catalyst concentration, when the increased light scattering reduces the photonic flux within the irradiated solution, thus lowering the degradation rate [29].

Preliminary experiments on the films immersed in water evidenced that, when increasing the percentages of titanium dioxide in the UV-curable formulation from 1 wt% to 3 wt%, the disappearance rate of 2,4,6-TCP during irradiation increases (data not shown). Therefore, thanks to the little influence of titanium dioxide on the kinetics of conversion, all the experiments presented below were done on the film of PEGDA containing 3 wt% of TiO_2. This film was immersed in water and subjected to irradiation and the production of hydrogen peroxide was measured. As shown in Figure 2, also in the presence of the polymeric film, hydrogen peroxide is rapidly formed and within 60 minutes of irradiation an amount of 7 μM is produced, which is even higher than that produced by TiO_2 in suspension.

Figure 2

Through the spectrophotometric assay with the DMAB-MBTH system, it was possible to evaluate the SBP enzymatic activity over time in the presence of different hydrogen peroxide concentrations. The results reported in Figure 3 evidence that the SBP activity remains similar for hydrogen peroxide concentrations ranging from 10^{-6} to 10^{-4} M, leading to the complete oxidation of the substrate (corresponding to $Abs_{590} \cong 1$). Therefore, as comes up from Figure 2, the amount of H_2O_2 produced during the irradiation of TiO_2 is enough to enable the enzymatic reaction.

Figure 3

3.3. Degradation of 2,4,6-trichlorophenol

On the bases of these results, 2,4,6-TCP was chosen as target molecule to test the potentiality of our photocatalytic/enzymatic system. Preliminary tests showed that the direct photolysis process is very slow and can be considered negligible in experiments carried out using 2 hours of irradiation time (data not shown).

3.3.1. System dispersed in aqueous solution

Upon light exposure, the disappearance of the 2,4,6-TCP occurs when titanium dioxide and SBP are dispersed in aqueous solution. As shown in Figure 4A, where the results are reported as a function of irradiation time, in the presence of SBP added with 1×10^{-4} M H_2O_2 a progressive, but slow, abatement of 2,4,6-TCP was observed (~ 17%, after 2 hours of irradiation), while in the presence of TiO_2 there is an almost complete disappearance of 2,4,6-TCP within 60 minutes. On the other hand, when SBP was added to the TiO_2 dispersion the reaction rate increases, and 2,4,6-TCP totally disappears in 30 minutes of irradiation; the reaction $t_{1/2}$ was reduced from 10 to 5 minutes.

Figure 4

The graph in Figure 4B shows the profiles of formation / disappearance of the transformation products identified in the presence of TiO_2 alone, or TiO_2 and SBP together. It should be noted that, in the presence of TiO_2 alone, the main intermediate species was phenol and 4-chlorophenol was also present, whereas in the system TiO_2/SBP, 4-chlorophenol was not observed and phenol concentration is significantly reduced.
In the case of the SBP/H_2O_2 system, any other intermediate was identified, but the reaction mixture initially turns from colourless to yellow, indicating the probable production of 2,6-dichloro-1,4-benzoquinone, in agreement with previous data reported for the reaction catalyzed by lignine peroxidase [30] and horseradish peroxidase [31].

3.3.2. System dispersed in polymeric film

A 2,4,6-TCP solution was subjected to irradiation in the presence of films containing optimized TiO_2 concentration (3 wt%) and increasing percentages of SBP. The degradation curves for film containing from 0.2 to 1.0 wt% SBP are plotted in Figure 5A. Films containing higher percentages of SBP (up to 2%) were also prepared, but not tested because they show loss of homogeneity and mechanical strength (local thickenings bring the film to disintegration when immersed in water).
The results shown in Figure 5A evidence that the immobilization of the enzyme in the film, and the long-term exposure to light, have not cleared the enzyme activity, since the disappearance rate increases with the increase of the SBP concentration. Although the process of immobilization reduces the activity of SBP to about 12% of that measured in solution (data not shown), from Figure 5A clearly appears that the addition to the system of

SBP, as in the case of suspensions, allows to increase the rate of degradation, which turns out to be the best photocatalytic system.

After 2 hours of irradiation, in the presence of films containing SBP (1 wt%)/H_2O_2 only 30% of 2,4,6-TCP is abated, while in the presence of TiO_2 (3 wt%) almost 50% of 2,4,6-TCP disappears. On the contrary, in the complete system containing TiO_2 (3 wt%) and SBP (1 wt%) the disappearance of the substrate is complete. It has to be underlined that the synergistic effect of photocatalytic and enzymatic action is particularly marked in the initial part of the process, when the reaction rate increasing well-matched with the kinetic curve of H_2O_2 production and with the maxima enzymatic activity, that was proved to be about 60% of the initial activity after 1 hour of irradiation. The effects of different irradiation times on the activity of SBP (both in solution and immobilized) are reported in Figure S1 of the supporting materials.

Still needs to be mentioned that, by subjecting the film to subsequent reaction cycles, a slow reduction of the activity with time was observed, as shown in Figure S2 of the supporting material. Nevertheless, about 93% of 2,4,6-TCP was degraded in the second reaction cycle after 120 min of irradiation, and 84% in the third cycle of reaction with the same sample of film.

Figure 5

The intermediates observed in the presence of the various experimental conditions are reported in Figure 5B. The presence of different conditions leads to the formation of diverse transformation products, which are also highlighted by the slower reaction rate respect to the experiments performed in aqueous suspension.

In the case of the polymeric film containing only SBP, no intermediates were observed, probably for the same reasons indicated above for the system dispersed in aqueous solution.

In the presence of TiO_2 only, the formation of 1,4-benzoquinone, as main transformation product, 2,6-dichlorophenol and phenol were observed, whereas, in the system containing both TiO_2 and SBP, only catechol and 1,4-benzoquinone were detected.

4. Conclusions

In the present work, we have studied a new photoactive material, able to remove water pollutants, obtained by TiO_2 and SBP dispersion in an acrylic UV-cured polymeric film. It was verified that the amount of hydrogen peroxide produced as a result of irradiation of titanium dioxide is capable of activating the catalytic mechanism of the enzyme peroxidase (SBP). This result made it possible to devise a system containing both the enzyme and the photocatalyst, where the active species were produced only by UV irradiation and are able to self-renew without the addition of external reagents. The use of the films has led to the operational advantages compared to solution, such as increased production of hydrogen peroxide.

For the different systems the efficiency of abatement was evaluated using the 2,4,6-TCP as model molecule. It is observed that SBP and TiO_2 act synergistically, leading to an increase in the rate for removal of the model molecules and coming to its complete degradation within 30 minutes, when used in aqueous suspension, and in 120 minutes when dispersed in film. Although the rate of 2,4,6-TCP removal is higher in suspension than in films, the dispersion of TiO_2 and SBP within a polymeric film allows to obtain a system able to be stable over time and easily reusable.

As recently remarked by Sumithran et al. [32], the oxidation of 2,4,6-TCP by plant peroxidases is the effect of two consecutive one-electron oxidations of the substrate *via* a phenoxy radical intermediate. Furthermore, Sturgeon et al. [33] have demonstrated by ESR spectroscopy that this species is rapidly converted in 2,6-dichloro-1,4-benzosemiquinone radical, which is not bound to the catalytic site and then undergoes the final rearrangement

to 2,6-dichloro-1,4-benzoquinone. It is therefore likely that these intermediates interact with the species produced by irradiation in the presence of TiO_2 (or are themselves substrates of the photocatalytic system) and could be the cause of the rapid and complete degradation of 2,4,6-TCP when it undergoes to the TiO_2/SBP system.

On the basis of our results, we are not able to define a complete degradation mechanism for 2,4,6-TCP, but some conclusions can be drawn. First of all, the experiments in aqueous solution show as the main reaction intermediate is phenol (Figure 4B), which can derive from a progressive reductive dechlorination of 2,4,6-TCP, as previously observed for TiO_2 or ZnO [12, 34], while the detection of 4-chlorophenol in the system without SBP can be due to a different initial process with formation of too unstable intermediates to be observed in turn in these experimental conditions.

Further, the system dispersed in a polymeric film has a slower action and a reduced circulation of substances between the catalyst and the solution, so it allows to observe other reaction intermediates (Figure 5B). For both the systems, with and without SBP, the main intermediate is 1,4-benzoquinone. The insertion of an oxygen atom is already observed in the reaction catalysed by SBP (as discussed above), while in the case of TiO_2 alone it can be supported by the proximity between titania and substrates, caused by the trapping into the polymeric matrix.

Finally, both in dispersion and in the film containing TiO_2/SBP, the degradation proceeds through the formation of less toxic intermediates than those produced by the system containing only TiO_2 because SBP is capable of activating the replacement of all the chlorine atoms present on the benzene ring through the formation of a phenoxy radical [35]. This finding is particularly interesting for future applications in the bioremediation field.

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Figure legends:

Figure 1: FESEM image of the fracture surface of the UV-Cured PEGDA resin containing 3 wt% of TiO_2.

Figure 2: Production of hydrogen peroxide from TiO_2 Degussa P25 as a function of irradiation time: when suspended in aqueous solutions (TiO_2 concentrations 0.2, 0.5 and 1.0 g L^{-1}) and immobilized in polymeric PEGDA film (containing 3 wt% TiO_2).

Figure 3: SBP activity respect to the MBTH-DMAB system as a function of hydrogen peroxide concentration: 1×10^{-4} M (solid line), 1×10^{-5} M (dashed line), and 1×10^{-6} M (dotted line).

Figure 4: (A), Degradation of 2,4,6-TCP (8×10^{-5} M) in the presence of SBP/H_2O_2 (1×10^{-8} M / 1×10^{-4} M), TiO_2 (1g L^{-1}) and TiO_2/SBP (1g L^{-1} / 1×10^{-8} M). (B) Intermediate species observed during 2,4,6-TCP degradation in presence of TiO_2 (upper) and TiO_2/SBP system (lower).

Figure 5: (A) Degradation of 2,4,6-TCP as a function of irradiation time in the presence of PEGDA films containing: SBP, TiO_2 3 wt%, and different amount of SBP. (B) Intermediate species observed during 2,4,6-TCP degradation in presence of TiO_2 (upper) and TiO_2/SBP system (lower).

Figure 1

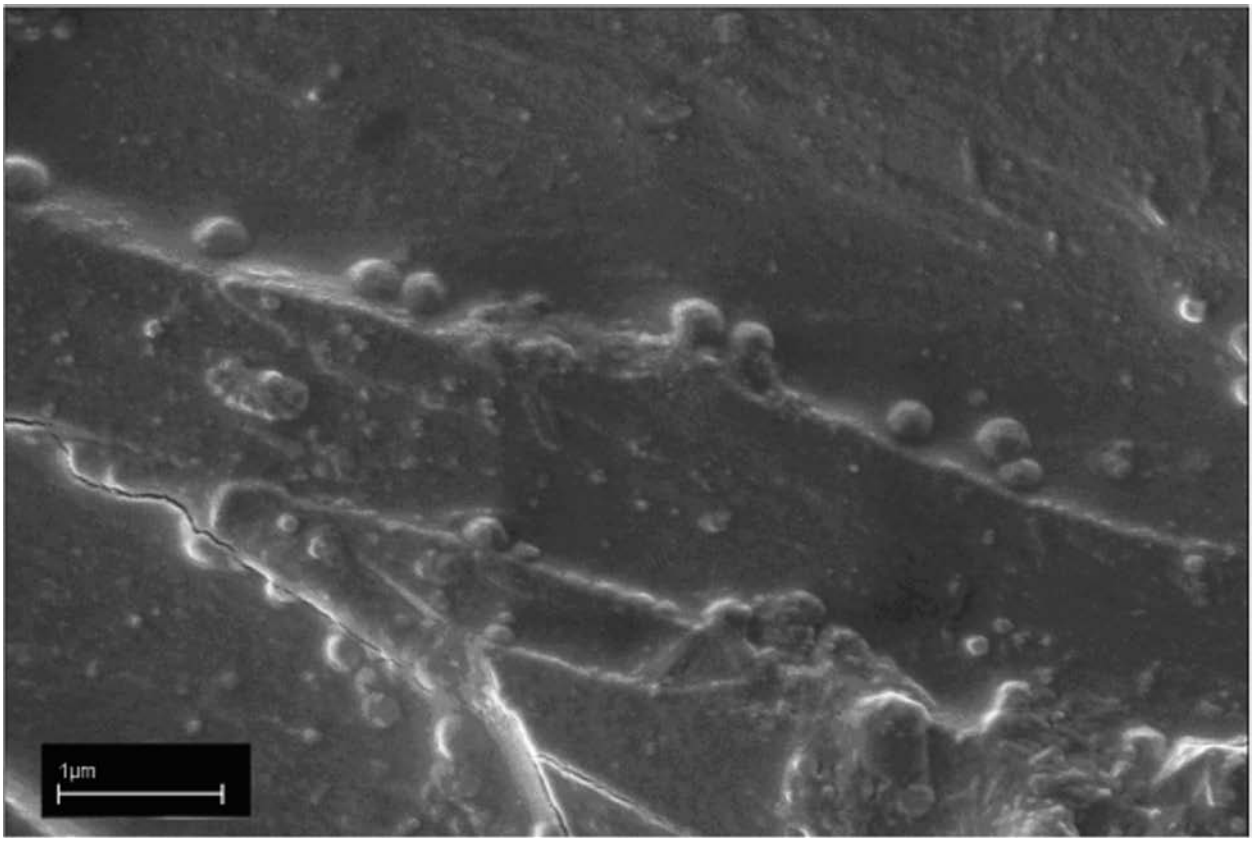


Figure 2

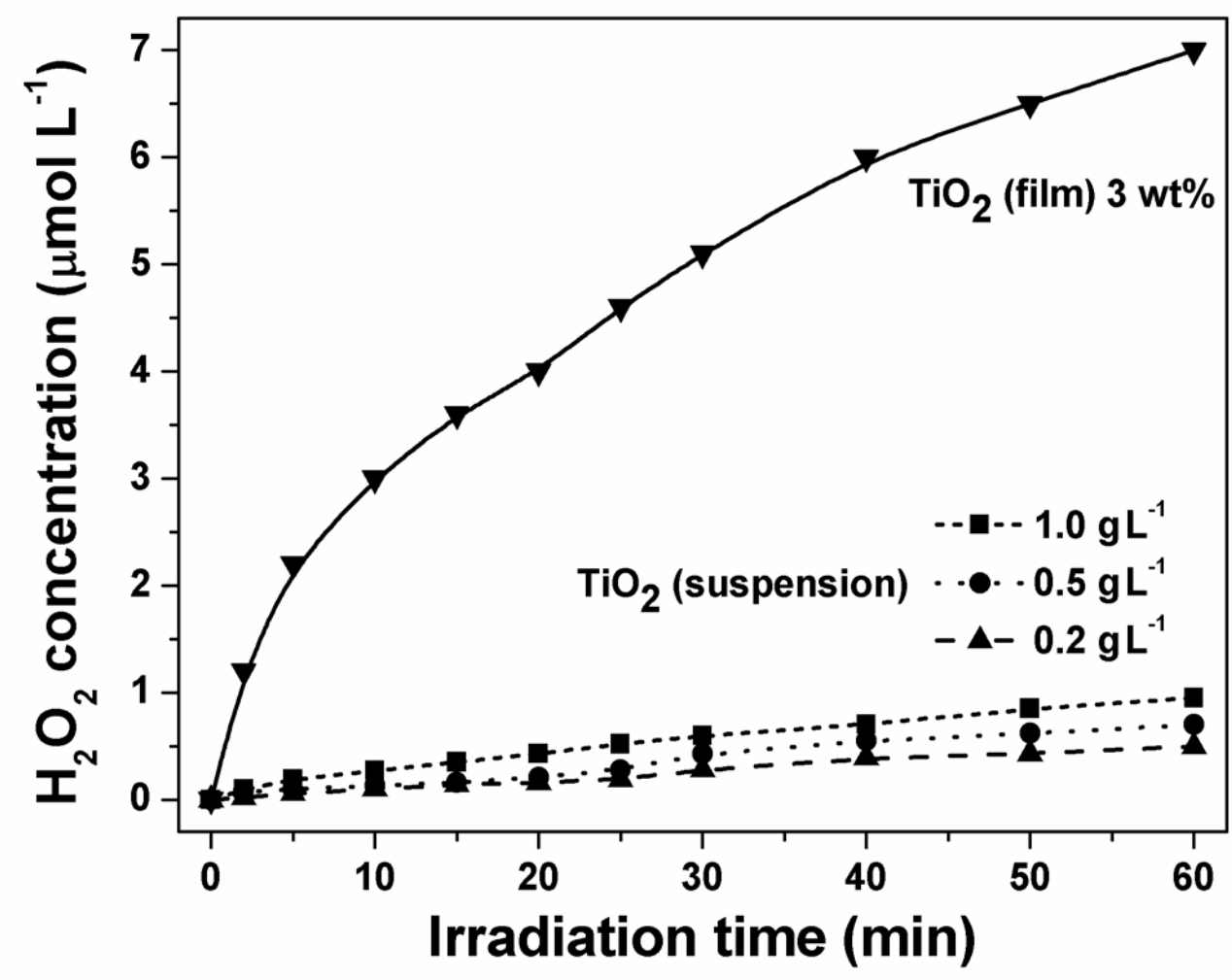

H2O2 concentration (μmol L-1)
Irradiation time (min)
TiO2 (film) 3 wt%
TiO2 (suspension)
1.0 gL-1
0.5 gL-1
0.2 gL-1
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60

Figure 3

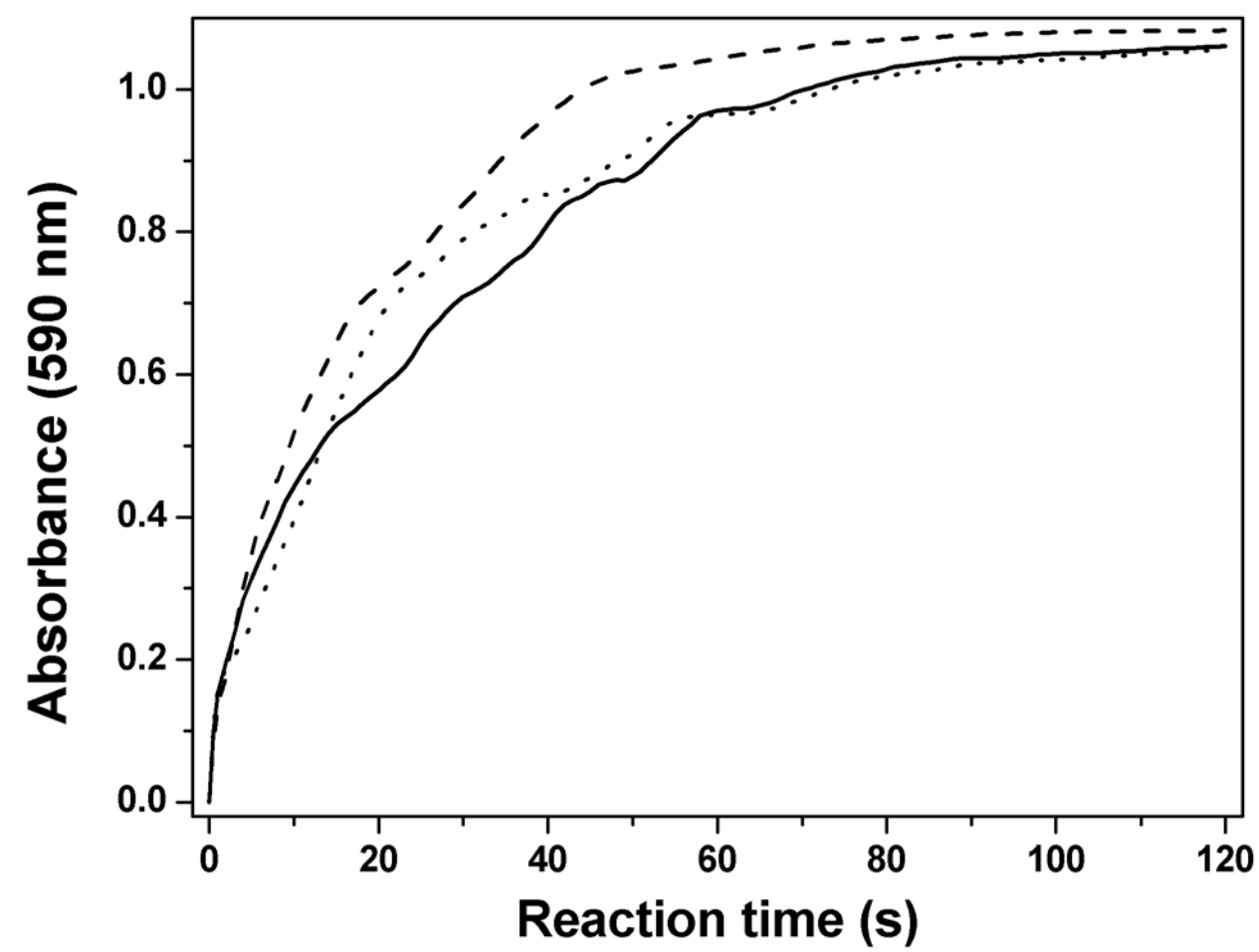
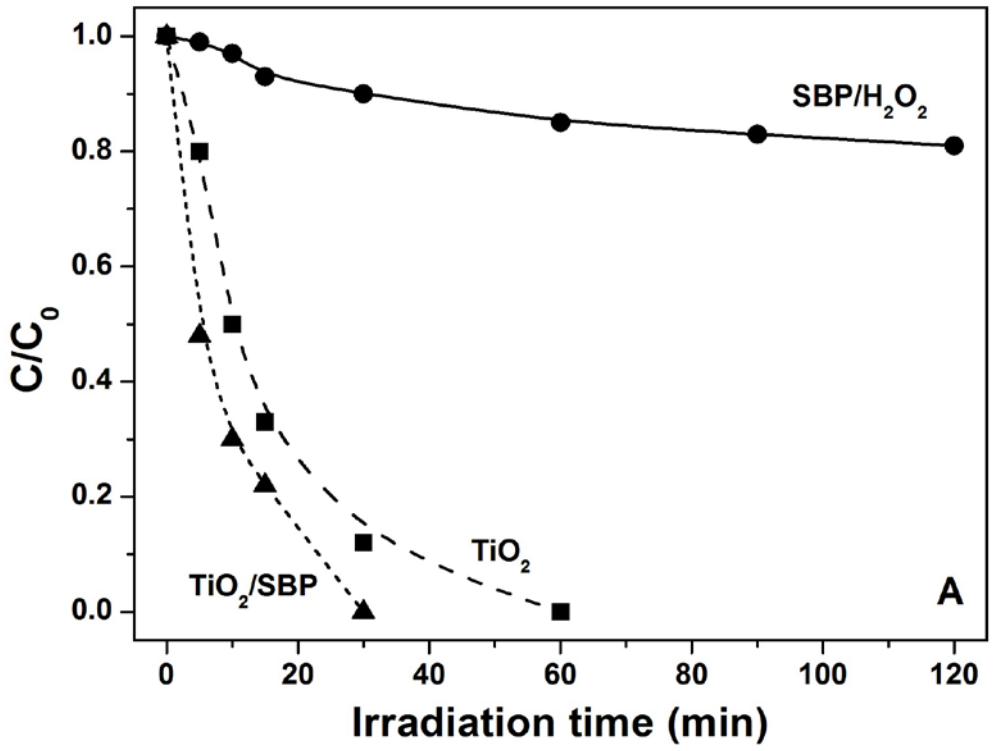

Absorbance (590 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Reaction time (s)

Figure 4


C/C0
1.0
0.8
0.6
0.4
0.2
0.0
SBP/H2O2
TiO2
TiO2/SBP
A
0
20
40
60
80
100
120
Irradiation time (min)

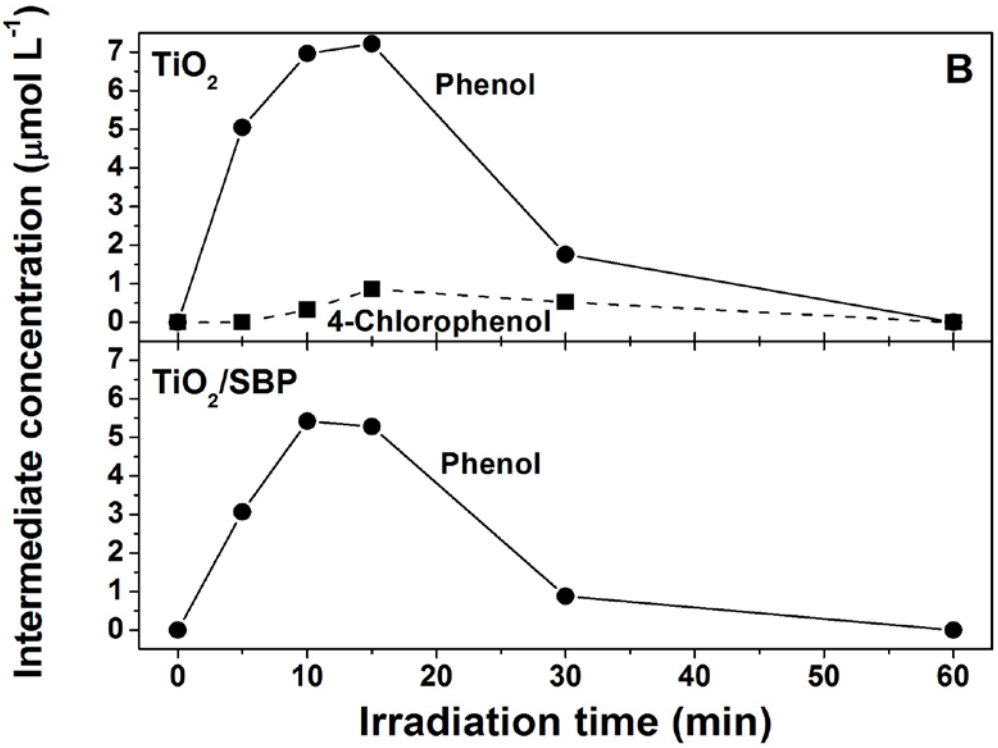
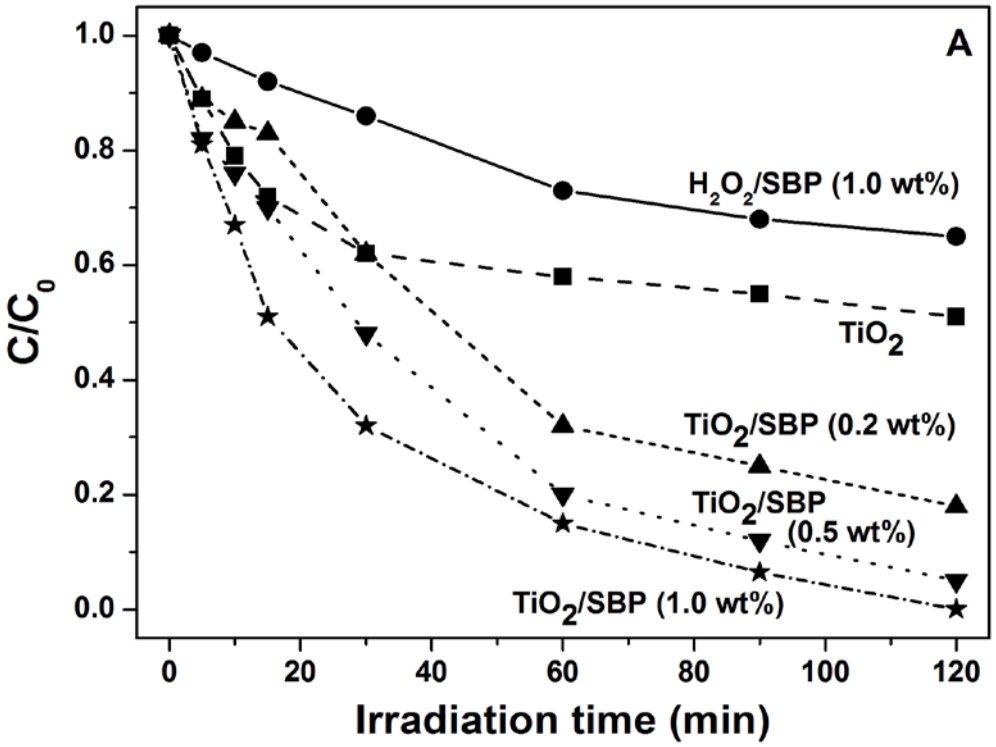
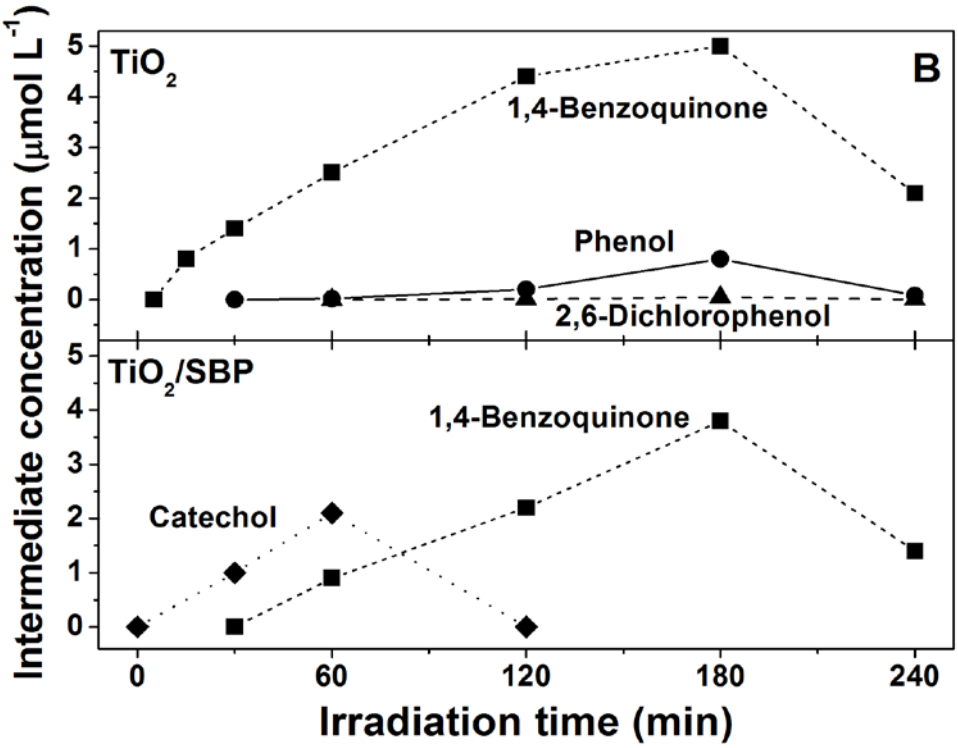

Intermediate concentration (μmol L-1)
TiO2
Phenol
B
4-Chlorophenol
TiO2/SBP
Phenol
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
Irradiation time (min)

Figure 5

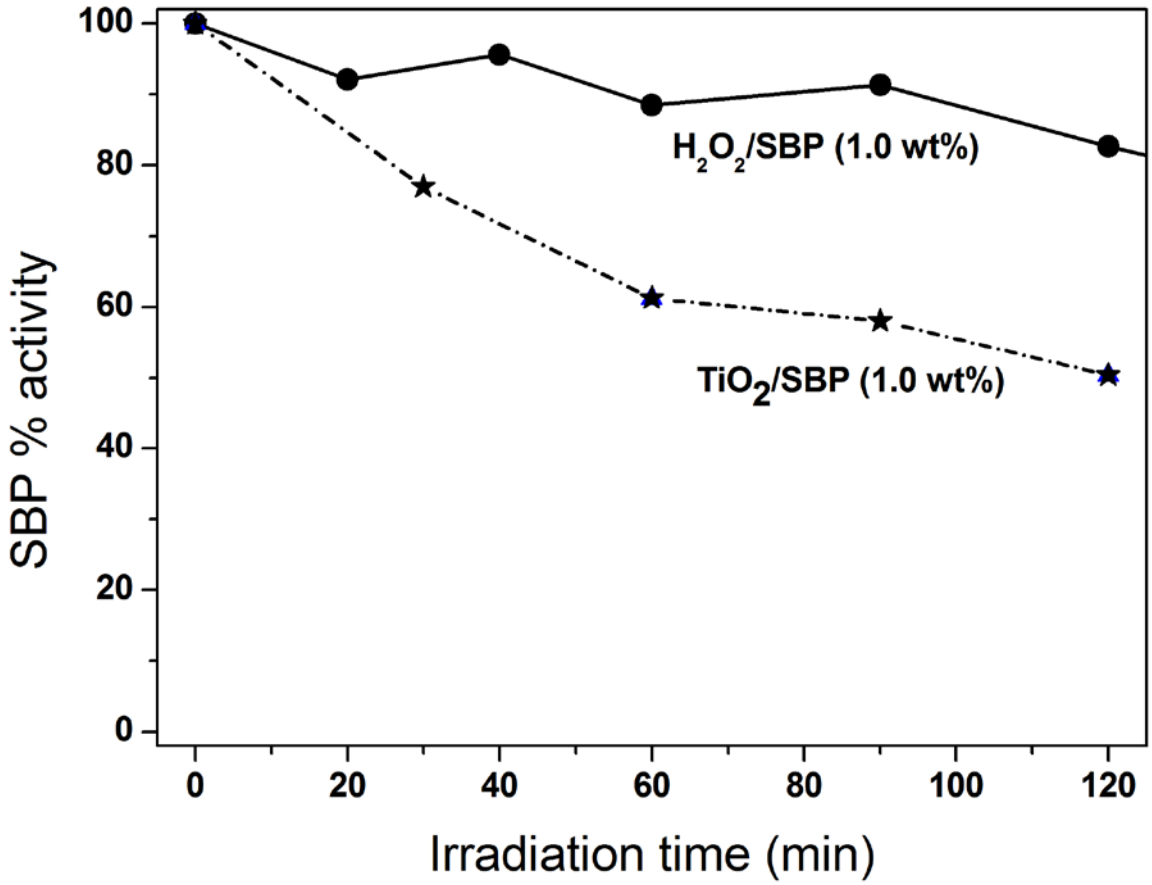

A
C/C0
Irradiation time (min)
H2O2/SBP (1.0 wt%)
TiO2
TiO2/SBP (0.2 wt%)
TiO2/SBP (0.5 wt%)
TiO2/SBP (1.0 wt%)

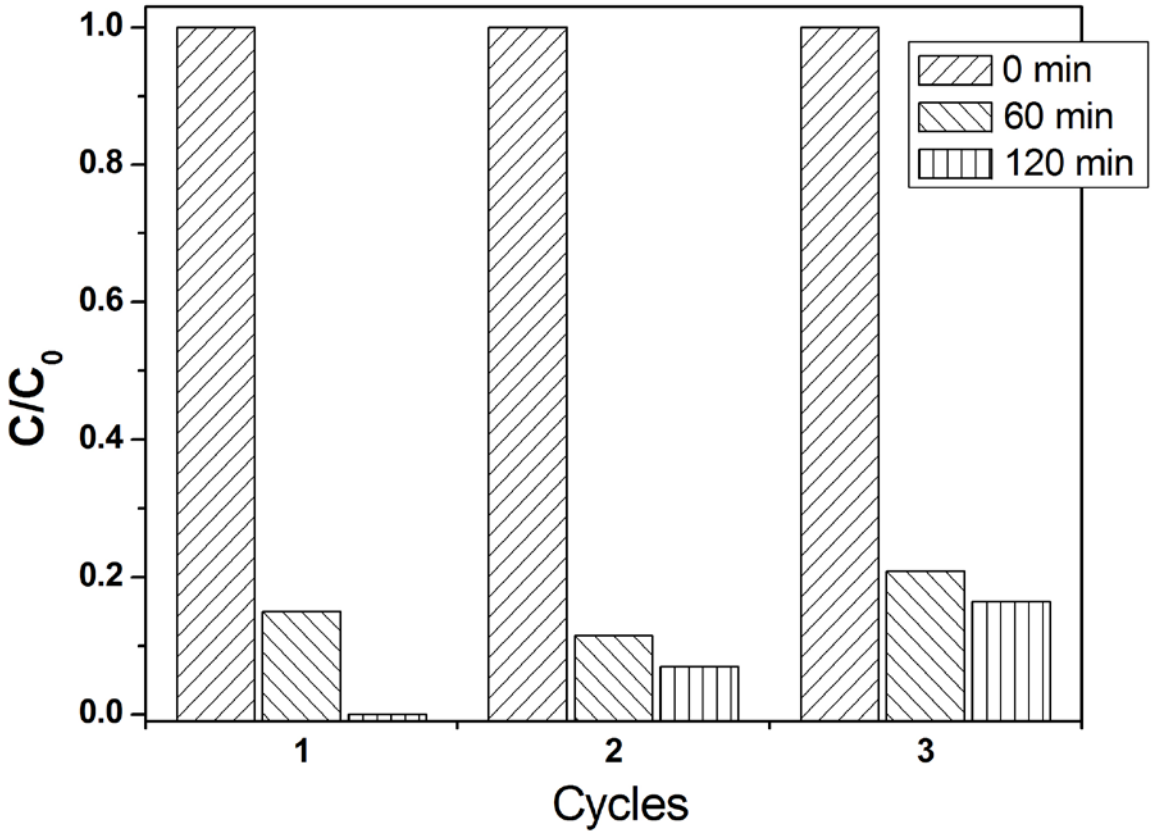

B
Intermediate concentration (μmol L-1)
TiO2
1,4-Benzoquinone
Phenol
2,6-Dichlorophenol
TiO2/SBP
1,4-Benzoquinone
Catechol
Irradiation time (min)

Supplementary material – Figure Legends

Figure S1: Relative activities, respect to the DMAB/MBTH system, for SBP in solution and immobilized in film as a function of the irradiation time.

Figure S2: Degradation of 2,4,6-TCP at different irradiation times. Data were obtained reusing for three times the same sample of film with TiO_2/SBP (1.0 wt%), data of cycle 1 are the same of Figure 5a.


SBP % activity
Irradiation time (min)
H2O2/SBP (1.0 wt%)
TiO2/SBP (1.0 wt%)
0
20
40
60
80
100
120


1.0
0.8
0.6
0.4
0.2
0.0
C/C0
1
2
3
Cycles
0 min
60 min
120 min